REVIEW

Systematic review of antitumour efficacy and mechanism of metformin activity in prostate cancer models

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Abstract

Metformin, the first line pharmacotherapy for type 2 diabetes has demonstrated favourable effects in prostate cancer (PCa) across a range of studies evaluating PCa patient outcomes amongst metformin users. However, a lack of rigorously conducted prospective studies has stalled clinical use in this setting. Despite multiple studies evaluating the mechanisms underpinning antitumour effects of metformin in PCa, to date, no reviews have compared these findings. This systematic review and meta-analysis consolidates the mechanisms accounting for the antitumour effect of metformin in PCa and evaluates the antitumour efficacy of metformin in preclinical PCa studies. Data were obtained through Medline and EMBASE, extracted by two independent assessors. Risk of bias was assessed using the TOXR tool. Meta-analysis compared in vivo reductions of PCa tumour volume with metformin. In total, 447 articles were identified with 80 duplicates, and 261 articles excluded based on eligibility criteria. The remaining 106 articles were assessed and 71 excluded, with 35 articles included for systematic review, and eight included for meta-analysis. The mechanisms of action of metformin regarding tumour growth, viability, migration, invasion, cell metabolism, and activation of signalling cascades are individually discussed. The mechanisms by which metformin inhibits PCa cell growth are multimodal. Metformin regulates expression of multiple proteins/genes to inhibit cellular proliferation, cell cycle progression, and cellular invasion and migration. Published in vivo studies also conclusively demonstrate that metformin inhibits PCa growth. This highlights the potential of metformin to be repurposed as an anticancer agent, warranting further investigation of metformin in the setting of PCa.

KEYWORDS
mechanism of action, meta-analysis, metformin, prostate cancer, systematic review

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Jeff Holst and Jennifer H. Gunter contributed equally.

1 | INTRODUCTION

Prostate cancer (PCa), the second most commonly diagnosed cancer amongst men worldwide,[1] has an incidence rate of almost 60% for men above 65 years.[1] The global mortality from PCa is estimated to double from 2018 to 2040, reaching 379 005 annual deaths.[1] These numbers indicate new therapies for advanced metastatic PCa are urgently needed.

The current management for locally advanced or metastatic PCa requires androgen deprivation therapy (ADT)[2,3]; however, ADT has limitations. While approximately 80% of PCa tumours respond to ADT,[4] 10–20% become refractory within 5 years.[5] This is known as castrate-resistant PCa (CRPC) and is defined by increasing PSA levels despite testosterone levels below 50 ng/dl.[3,6] The median prognosis for CRPC is 2 years.[7] In addition, ADT has several adverse metabolic and cardio-vascular effects[8] including rapid development of insulin resistance, increased cholesterol, sarcopenic obesity[9] and an increased risk of diabetes mellitus.[10] By removing an important regulator of prostate differentiation, ADT also facilitates epithelial to mesenchymal transition (EMT), a key process underlying metastasis and therapeutic resistance.[11] AR-signalling inhibitors (ARSIs) such as abiraterone and enzalutamide have been widely adopted for CRPC, in line and in combination with chemotherapies such as docetaxel. However, these drugs only extend survival by a matter of months compared with placebo.[12] This is partially due to the development of resistance[13] which highlights the need for new therapeutic strategies that counteract drug resistance and mitigate the significant side effects of the current therapeutics.

Metformin as first-line pharmacotherapy for type 2 diabetes mellitus (T2DM) patients is used by approximately 120 million people worldwide[14] with no significant long-term safety issues identified.[15] Apart from improving insulin sensitivity, metformin has cardiovascular benefits such as reducing cholesterol levels[16] and body weight[15] and may counteract ADT-induced adverse cardio-metabolic effects. Various epidemiological studies have shown diabetes patients treated with metformin had reduced risk of cancer, including PCa compared with patients on other antiglycaemic agents such as insulin and secretagogues such as sulphonylureas[17–22] with improved survival outcomes.[23,24] In contrast, other studies have shown metformin does not significantly reduce the risk of PCa.[22,25,26] Therefore, multiple systematic reviews and meta-analyses have been conducted to evaluate the clinical outcome of metformin users, specifically evaluating PCa risk (Table 1) and survival (Table 2). With the exception of three studies,[27–29] most meta-analyses have indicated metformin does not reduce the risk of PCa (Table 1).[30–34] However, a significant number of observational studies included in these meta-analyses were retrospective, which may have significant bias. When only considering prospective studies, there was a slight reduction in PCa risk with metformin (summary relative risk = 0.93; 95% CI: 0.89–0.97).[29] In terms of clinical outcome, five meta-analyses conclusively indicated that metformin reduced biochemical recurrence (BCR) of PCa,[28,35–38] highlighting the possibility of repurposing this antidiabetic drug as an antitumour agent in PCa (Table 2).

Multiple in vitro and in vivo studies have investigated the mechanisms accounting for the antitumour effect of metformin in PCa,[39–41] but to date, no reviews have consolidated these findings. Hence, this systematic review and meta-analysis aims to consolidate the published mechanisms accounting for the antitumour effect of metformin in PCa and evaluate the antitumour efficacy of metformin in preclinical PCa studies.

2 | METHODS

2.1 | Search strategy

The search strategy was based on population intervention comparator outcome (PICO) format: What is the cell intrinsic and whole metabolic effects (Outcome) of metformin (Intervention) on prostate cancer (Population)? This systematic review was carried out following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. N.F.W and J.H.G searched for articles using two search engines: Embase and Medline. Articles published in these search engines up to the date 20 May 2020 were screened. We started with a broad search strategy (Tables S3 and S4), including search terms such as "metformin", "prostate cancer", "anti-proliferative", "anti-tumour", "tumour recurrence", "metabolism", "risk", "morbidity", and "mortality". The initial aim was to correlate the antitumour effect of metformin from in vitro studies with clinical outcomes of PCa patients on metformin. Reports containing only clinical studies, without basic or translational components, were excluded as there have been a multitude of systematic reviews evaluating this outcome.[27,30,32,33,35–38]

2.2 | Study selection

2.2.1 | Inclusion criteria

This review included all studies that investigated mechanisms behind the antitumour effect of metformin in PCa cell lines, mouse models, and in PCa patients.

2.2.2 | Exclusion criteria

This review excluded the following articles (Figure 1):

1. Studies with incorrect intervention.
2. Articles with incorrect/missing outcomes.
3. Studies with incorrect tumour type.
4. Clinical studies of patient's outcome following metformin treatment.
5. Studies investigating metformin effect on standard/non-standard prostate cancer treatment.
6. Conference abstracts, reviews, notes or editorials.
7. Non-English language publications.

TABLE 1 Summary of systematic review/meta-analyses evaluating metformin effect on PCa risk. Results were obtained from clinical data of diabetic patients

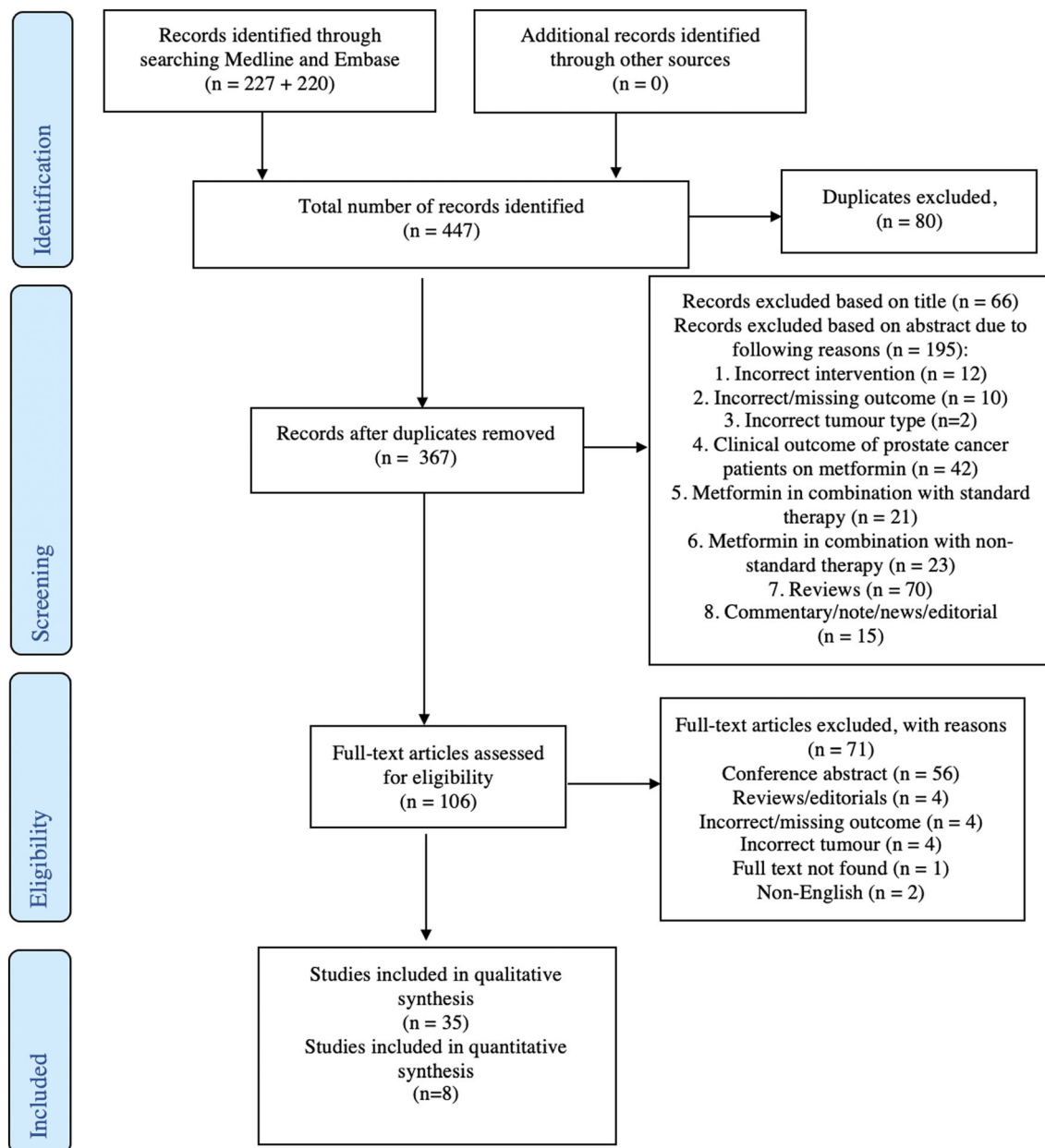| Author	Year	Title	Statistics	Risk of PCa
DeCensi et al.[25]	2010	Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis	RR: 0.69, 95% CI: 0.61–0.79 p value: 0.03	↓
Franciosi et al.[28]	2013	Metformin therapy and risk of cancer in patients with type 2 diabetes: systematic review	OR: 1.18 95% CI: 0.69–2.04 p value: 0.545	NS
Zhang et al.[29]	2013	Association of metformin use with cancer incidence and mortality: a meta-analysis	RR: 0.91 95% CI: 0.80–1.03	NS
Yu et al.[26]	2014	Effect of Metformin on Cancer Risk and Treatment Outcome of Prostate Cancer: A Meta-Analysis of Epidemiological Observational Studies	OR: 0.91 95% CI: 0.85–0.97 p- value: 0.014	↓
Gandini et al.[27]	2015	Metformin and Cancer Risk and Mortality: A Systematic Review and Meta-Analysis taking into account Biases and Confounders	SRR: 0.93 95% CI: 0.89–0.97	↓
Chen et al.[30]	2018	Metformin, Asian ethnicity and risk of prostate cancer in type 2 diabetes: a systematic review and meta-analysis	RR: 1.01 95% CI: 0.8–1.28 p value: 0.92	NS
Feng et al.[31]	2019	Metformin use and prostate cancer risk: A meta-analysis of cohort studies	RR: 0.97 95% CI: 0.8–1.16 p value: 0.711	NS
Ghiasi et al.[32]	2019	The Relationship Between Prostate Cancer and Metformin Consumption: A Systematic Review and Meta-analysis Study	OR: 0.89 95% CI: 0.67–1.17	NS

Note: NS: No statistically significant relationship between metformin and risk of PCa; RR: relative risk; SRR: summary relative risk; OR: odds ratio, ↓: metformin reduces risk of PCa.

TABLE 2 Systematic review/meta-analyses summary of metformin effect on PCa patient outcome

Author	Year	BCR	Overall survival	Cancer specific mortality	Cancer specific survival	All-cause mortality
Yu et al.[26]	2014	↓ HR: 0.81 95% CI: 0.68–0.98 p value: 0.014	-	-	-	NS HR: 0.86 95% CI: 0.64–1.14 p value: 0.001
Hwang et al.[33]	2015	RR*:1.20 95% CI*: 1–1.44	-	NS RR*: 2.27 95% CI*: 0.61–8.38	-	NS RR*: 1.26 95% CI*: 0.75–2.12
Raval et al.[34]	2015	↓ HR: 0.82 95% CI: 0.67–1.01 p value: 0.06	-	NS HR: 0.76 95% CI: 0.43–1.33 p value: 0.33.	-	NS HR: 0.86 95% CI: 0.67–1.1 p value: 0.23
Coyle et al.[35]	2016	↓ HR 0.83 95% CI: 0.69–1.00	Improved HR: 0.82 95% CI: 0.73–0.93.	-	Improved HR 0.58 95% CI: 0.37–0.94	-
Stopsack et al.[36]	2016	↓ HR: 0.79 95% CI: 0.63–1.00 p value = 0.047	Improved HR: 0.88 95% CI: 0.86–0.90 p value < 0.001	NS HR 0.76 95% CI: 0.44–1.31 p value = 0.33		-

Note: ↓: Reduced biochemical recurrence. NS: No significant statistical relationship between metformin use and outcome. -: Outcome not investigated in study.
*Statistical results show results of PCa patients with T2DM without the use of metformin.

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Identification
  Records identified through          Additional records identified
  searching Medline and Embase        through other sources
  (n = 227 + 220)                     (n = 0)

                    Total number of records identified          Duplicates excluded,
                    (n = 447)                                   (n = 80)

Screening
                    Records after duplicates removed    Records excluded based on title (n = 66)
                    (n = 367)                           Records excluded based on abstract due to
                                                        following reasons (n = 195):
                                                          1. Incorrect intervention (n = 12)
                                                          2. Incorrect/missing outcome (n = 10)
                                                          3. Incorrect tumour type (n=2)
                                                          4. Clinical outcome of prostate cancer
                                                             patients on metformin (n = 42)
                                                          5. Metformin in combination with standard
                                                             therapy (n = 21)
                                                          6. Metformin in combination with non-
                                                             standard therapy (n = 23)
                                                          7. Reviews (n = 70)
                                                          8. Commentary/note/news/editorial
                                                             (n = 15)

Eligibility
                    Full-text articles assessed         Full-text articles excluded, with reasons
                    for eligibility                     (n = 71)
                    (n = 106)                           Conference abstract (n = 56)
                                                        Reviews/editorials (n = 4)
                                                        Incorrect/missing outcome (n = 4)
                                                        Incorrect tumour (n = 4)
                                                        Full text not found (n = 1)
                                                        Non-English (n = 2)

Included
                    Studies included in qualitative
                    synthesis
                    (n = 35)
                    Studies included in quantitative
                    synthesis
                    (n=8)
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FIGURE 1 PRISMA flow chart for study inclusion.

2.2.3 | Data extraction

The authors devised a search strategy to include all possible related synonyms (Tables S3 and S4), identifying 367 articles after duplicates were removed. These articles were screened by both N.F.W and J.H. G based on title, and then abstract and 35 articles were included in this systematic review (Figure 1). Any disagreement in exclusion criteria was resolved via discussion.

Both authors extracted the following data simultaneously from each article: title, author names, year of study, as well as the study objective, outcomes, and methodology. Similar data was collated by both authors.

2.3 | Risk of bias

To evaluate risk of bias in each article, the Toxicological data Reliability (TOXR) Assessment Tool was used. An 18-point and 21-point assessment was evaluated for in vitro and in vivo studies, respectively. The TOXR tool categorizes each article into three different categories: 1, 2, or 3, based on minimum criteria. Category 1 articles are reliable without restrictions; Category 2 articles are reliable with restrictions; Category 3 articles are unreliable and not to be used as a key study. Both authors assessed the risk of bias of each article individually, and discrepancies resolved via discussion.

2.4 | Statistical analysis

Meta-analysis was performed on in vivo data to evaluate the effect of metformin treatment on tumour volume. All data were extracted from article texts, tables, and figures, with any estimates made based on the presented data and figures, where tumour volume for control and experimental groups were reported (mean $\pm$ standard deviation mm³) or could be derived. Derived data included variance estimations based on established statistical methodologies. Statistics were performed on continuous data from 16 experiments from eight studies[40-47] using random effects model to pool effects sizes for meta-analysis. Standardized mean difference (Cohen's *d*) was calculated in RevMan software. Heterogeneity was tested using I^2 statistic. Publication bias was evaluated using funnel plot analysis.

3 | RESULTS

3.1 | Study selection and characteristics

A total of 447 articles were identified from Medline and Embase, which included 80 duplicated articles. We screened 367 unique records based on the article title, resulting in 66 articles excluded, which did not match study characteristics. The remaining 301 articles were subjected to screening based on the abstract, with 195 records excluded due to reasons outlined in Figure 1. The full text of the remaining 106 articles was assessed, with a further 71 articles excluded (Figure 1), the majority (56) being conference abstracts. Thus, 35 articles remained for qualitative analysis, and of these, eight studies evaluated the efficacy of metformin in reducing PCa tumour growth in mice models and were included for quantitative analysis (Figure 1).

The 35 articles were published between 2003 and 2018, showing sustained interest in this field. Of the 35 articles included for qualitative analysis, 31 investigated the antitumour effects of metformin using PCa cell lines, 13 articles included mouse models, and five articles included PCa patients. The outcomes of these studies are summarized in Table S5. Out of the 13 articles that included mouse models, eight articles evaluated subcutaneous PCa xenograft tumour volume following metformin administration and utilized similar dosing and experimental duration. The remaining five in vivo articles were excluded as they did not report tumour volume and were instead evaluating metastasis or protein expression.

3.2 | Qualitative analyses

3.2.1 | Risk of bias

The TOXR tool evaluates the reliability of in vitro and in vivo data reporting, assigning weighted scores in the categories of drug identification, in vitro or in vivo model characterization, study design and documented results, with in vivo studies also assessed for plausibility of study design and results. Each criterion within a category is given a score of 1 if the paper contained each relevant detail, or a score of 0 if details were omitted. The 18-point system from the TOXR tool was used to evaluate all 31 in vitro studies.[48] Category 1 articles scored 15 or more out of 18, category 2 scored between 11 and 14, and a score of 11 or the inability to meet critical criteria was a category 3 article. Only two of 31 in vitro studies were categorized as category 3 as the critical criterion were not met (Table S6).[42,49] Caraci et al. did not state the metformin concentration used,[49] whereas Chen et al.'s study design was inappropriate in achieving the specific aims as it did not evaluate PEDF knockdown or gene inhibition on PCa proliferation, migration, and tumour growth.[42] Therefore, the findings from these category 3 articles were classified as unreliable. Three articles were assigned to category 2[50-52] as multiple minor criteria were not met including purity of substance,[50-52] experimental incubation temperature and percentage of CO_2,[50,51] number of cells seeded,[50] statistical tests performed,[50,52] and number of replicates in each experiment.[51,52] The remaining 26 studies were classified as category 1 and deemed reliable for further analysis (Table S6).[39,40,43,44,46,47,53-71]

The 21-point system from the TOXR tool was used to evaluate the 13 in vivo mouse studies. Only one of the 13 studies was classified as category 3 as not all critical criteria were met.[72] The study design was inappropriate in achieving the aim as mice did not harbour PCa tumours, despite the study hypothesising metformin may interfere with PCa progression.[72] An additional four studies were classified as category 2[44,45,55,68] with a score between 13 and 17. Criteria that were not met included purity of substance,[44,45,55,68] gender of organism,[44,55,68] age/weight of organism,[44,45,55] feeding/housing conditions,[45,55] statistical significance,[44,45,68] source of metformin,[68] and vehicle used to dissolve metformin.[68] The remaining eight studies were classified as category 1, meeting most criteria with a score of 18 or more.[40-43,46,47,54,62]

Of the 35 articles identified, five articles evaluated the clinical effects of metformin in human PCa patients. Again, the 21 point system from the TOXR tool was used to evaluate each article, with three articles classified as category 3 as critical criteria such as metformin dose,[55,68] or frequency and duration of exposure to metformin was not disclosed,[55,68,73] while the remaining two articles were category 1 articles with most/all criteria being met[47,74] (Table S6).

3.2.2 | Type of outcomes

The outcomes most reported for in vitro studies included cell viability, apoptosis, migration, invasion, cell cycle arrest, and protein expression. For in vivo studies, the outcomes included tumour volume, and in a small number of studies, metastatic burden (Table S5).

3.2.3 | Cell growth

Major hallmarks of cancer include ability to evade cell death and sustaining proliferative signals.[75] To evaluate the antitumour effect of

metformin, one of the most reported outcomes in the 31 in vitro studies was the effect of metformin on PCa cell viability. The Cell Counting Kit-8 assay, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and related MTS and Alamar blue assays were used to evaluate cell viability. The caveat of these assays is they extrapolate viability from mitochondrial activity and metformin is a known inhibitor of Complex I of the electron transfer chain. Decreased mitochondrial respiration may result in an overestimation of the effects of metformin on cell viability using these approaches.

The extent of inhibition on cell proliferation was dependent on the experimental method (e.g., incubation period, concentration of drug, and cell line used). A range of doses from 0.1–50 mM were tested, with 5 mM the most common concentration, still well above the reported peak-plasma concentrations obtainable in humans,[76] with four studies using concentrations in the high micromolar range.[42,45,46,70] The viability of androgen-dependent PCa cell lines (LNCaP, VCaP, DuCaP) was evaluated in 15 studies and showed significant inhibition of cell growth (Table S5).[39–42,46,50–52,55,58–61,64,67] LNCaP cells treated with 5 mM metformin for 48 h resulted in growth inhibition between 25% and 60% (Table S5).[41,46,50,51,59,64] There were 20 in vitro studies ($n = 20$) that evaluated viability using androgen-independent PCa cell lines (DU145, PC-3, C4-2, PPC1, LNCaP-LA, 22Rv1), most commonly PC-3 and DU145.[39–42,44,46,47,50–55,59–61,64,66,67,70] Metformin reduced cell viability of androgen-independent PCa cells in 17 out of 20 studies,[39,40,42,44,46,47,50–55,59–61,66,67] whereas three studies showed a nonsignificant effect.[41,64,70] Lastly, six in vitro studies evaluated the outcome of metformin on the cell viability of benign prostatic epithelial cells (PNT1A, RWPE-1, EP156T, P69),[39–41,54,61,70] which reported no effect in three out of six studies (50%),[54,61,70] but growth inhibition ranging from 20% to 60% (treated for 24, 72, and 96 h) in the remaining 3.[39–41] In addition, both Wang et al. and Shen et al. showed the combination of metformin and ARSI, bicalutamide resulted in greater inhibition on PCa cell viability compared with either agent alone.[52,59]

3.2.4 | Apoptosis

Nine studies evaluated whether reduced viability with metformin was due to apoptosis. Levels of apoptosis were evaluated by flow cytometry and annexin V staining or caspase 3 cleavage.[40,42,44,46,47,52,54,59,70] All of the studies included androgen-independent PCa cell lines (C4-2B, PC-3, CWR22Rv1, DU145),[40,42,44,46,47,52,54,59,70] whereas three studies included androgen-dependent (LNCaP) cell lines,[40,42,59] and one study included the benign prostatic cell line PNT1A.[70] The effect of metformin on induction of apoptosis in PCa cells remains relatively controversial, as three out of the nine studies showed that metformin (0.1–10 mM) does not significantly induce apoptosis in androgen-independent PCa cell lines,[40,46,70] whereas the remaining six that assessed higher metformin concentrations of 2–30 mM showed increased induction of apoptosis.[42,44,47,52,54,59] Of the three studies that evaluated metformin and apoptosis of LNCaP cells, one

study using 5-mM metformin showed a nonsignificant effect,[40] whereas two studies using metformin (0.625–30 mM) showed increased induction of apoptosis following metformin treatment.[42,59]

3.2.5 | Cell cycle inhibition

Cell cycle is a tightly regulated process in most functioning cells with multiple checkpoints that trigger cell cycle arrest. However, cancer cells have deregulated cell cycle and increased proliferation.[77] A total of eight in vitro studies evaluated the effect of metformin on PCa cell cycle progression. The most used method to evaluate cell cycle was flow cytometry.[40,42,47,50,54,58,61,66] A range of PCa cell lines were used in these studies: Four studies used only androgen-dependent PCa cell lines (LNCaP, VCaP),[40,50,58,61] three studies used only androgen-independent PCa cell lines (C4-2B, 22Rv1, DU145, PC-3),[47,54,66] and one study used both.[42] The concentration range of metformin ranged from 0.625 to 10 mM, with 5 mM most commonly used. In six of the eight studies, cell cycle progression was inhibited in both androgen-independent and androgen-dependent PCa cell lines (LNCaP, VCaP, C4-2B, 22Rv1, DU145) with an increased percentage of cells in G_0/G_1 phase or reduced percentage of cells in $S + G_2/M$ phase (Table S5).[40,50,54,58,61,66] For PCa cell lines treated with 5 mM metformin, there was a reduction of 7.1–16.2% of cells in S-phase following treatment, and an increase of 9.1–24% of cells in G_0/G_1 (Table S5).[40,50,61,66] The variation in percentage reduction could be due to the difference in PCa cell type and/or incubation period. Liu et al. mentioned possible inhibition in PC-3 and DU145 cell cycle progression by metformin; however, the percentage reduction/increment was neither given in the text nor the supplementary figure.[47] In contrast, Kato et al. showed 24-h exposure to metformin (0.625, 2.5, and 10 mM) did not alter cell cycle progression in either PC-3 or LNCaP cells.[46]

3.2.6 | Cellular invasion and migration

The invasive and migratory ability of PCa cells is critical in the development of metastasis.[78] Thirteen studies evaluated the effect of metformin on PCa cell migration/invasion,[39,41–43,46,47,58,61,62,66,68–70] typically using Boyden's chamber or wound healing assays, in androgen-dependent (LNCaP, VCaP), and androgen-independent PCa cell lines (PC-3, DU145, 22Rv1) and benign prostate epithelial cells (PNT1A and RWPE). All five studies that evaluated the effect of metformin on migration and/or invasion of androgen-dependent PCa cells concluded metformin (0.625–20 mM) reduced cellular migration and/or invasion in androgen-dependent PCa cell lines.[39,42,47,58,61] For androgen-independent PCa cell lines, eight of nine studies that evaluated cellular migration using metformin (1–20 mM)[39,41–43,46,66,68,69] and six out of seven that evaluated cellular invasion using metformin (0.625–20 mM),[42,43,46,62,68,69] showed similar findings. Therefore, the inhibitory effect of metformin on cellular migration and invasion may be independent of the androgen receptor (AR) signalling pathway.

3.2.7 | Protein expression and molecular pathways

Since Evans et al. first reported in 2005 that metformin use in T2DM patients was associated with a reduced risk of cancer,[79] multiple pre-clinical studies have been carried out to delineate its molecular mechanisms. The hallmarks of cancer include the ability of maintaining proliferative signalling, evasion of growth suppressors, evasion of apoptosis, inducing angiogenesis, invasion and metastasis, and lastly allowing for indefinite cellular replication.[75] Four emerging hallmarks have emerged including reprogramming energy metabolism to allow for rapid cell growth and division, and the ability to evade immune destruction.[75] From the 31 in vitro studies identified, it appears metformin inhibits a large proportion of these capabilities, summarized in Figures 2 and 3.

Activation of AMP activated kinase (AMPK) is a widely known effect of metformin, which inhibits several metabolic enzymes such as mammalian target of rapamycin (mTOR), and acetyl-CoA carboxylase (ACC), responsible for protein synthesis and fatty acid synthesis respectively (Figure 2).[40,63] Both of these processes are commonly elevated in cancer cells.[80] Therefore, activation of AMPK by metformin could account for one of its antitumour mechanisms, affecting energy triage and controlling cell cycle, biomass accumulation for cell proliferation and apoptosis. Of the 31 in vitro articles, 11 studies evaluated PCa cell lines for AMPK activation by western blot. These studies consistently reported metformin results in increased pAMPK expression while total AMPK levels remained relatively unchanged.[40,44,50,51,53,57,59,60,65,66,70]

Apart from inhibiting ACC and mTORC, the activation of AMPK by metformin has also been associated with the reduction of FoxM1, a transcription factor that regulates EMT, vital for PCa cellular migration.[60] Knockdown of FoxM1 by shRNA in androgen-independent DU-145 cells resulted in reduced cellular migration, decreased expression of mesenchymal markers (vimentin and slug) and increased expression of epithelial markers (E-cadherin).[60] These data suggest that metformin may inhibit EMT, a process underlying metastasis and therapeutic resistance, via suppression of FoxM1 in PCa.[60] Several additional studies have linked metformin with inhibition of EMT in PCa, although whether these pathways are AMPK-dependent has not been investigated. For instance, metformin treatment resulted in an upregulation of miR30a (a tumour suppressor) and subsequent reduction of SOX4 (oncogenic transcription factor), a known EMT regulator (Figure 3).[61] Metformin treatment also inhibits the phosphorylation and oxidation of pyruvate kinase M2 (PKM2), thereby preventing the downregulation of miR205, a negative regulator of EMT.[62] In addition, while ADT is the gold standard treatment for PCa, ADT induces EMT possibly through the upregulation of COX2 and p-STAT3.[68] Metformin was shown to reduce expression of COX-2, PGE2, and p-STAT3, potentially inhibiting castration-induced EMT.[68] Finally, metformin, only at very high concentrations, were shown to increase the tumour suppressor miR-101 that downregulates expression of glyoxalase 1, which also inhibits EMT (Figure 3).[69]
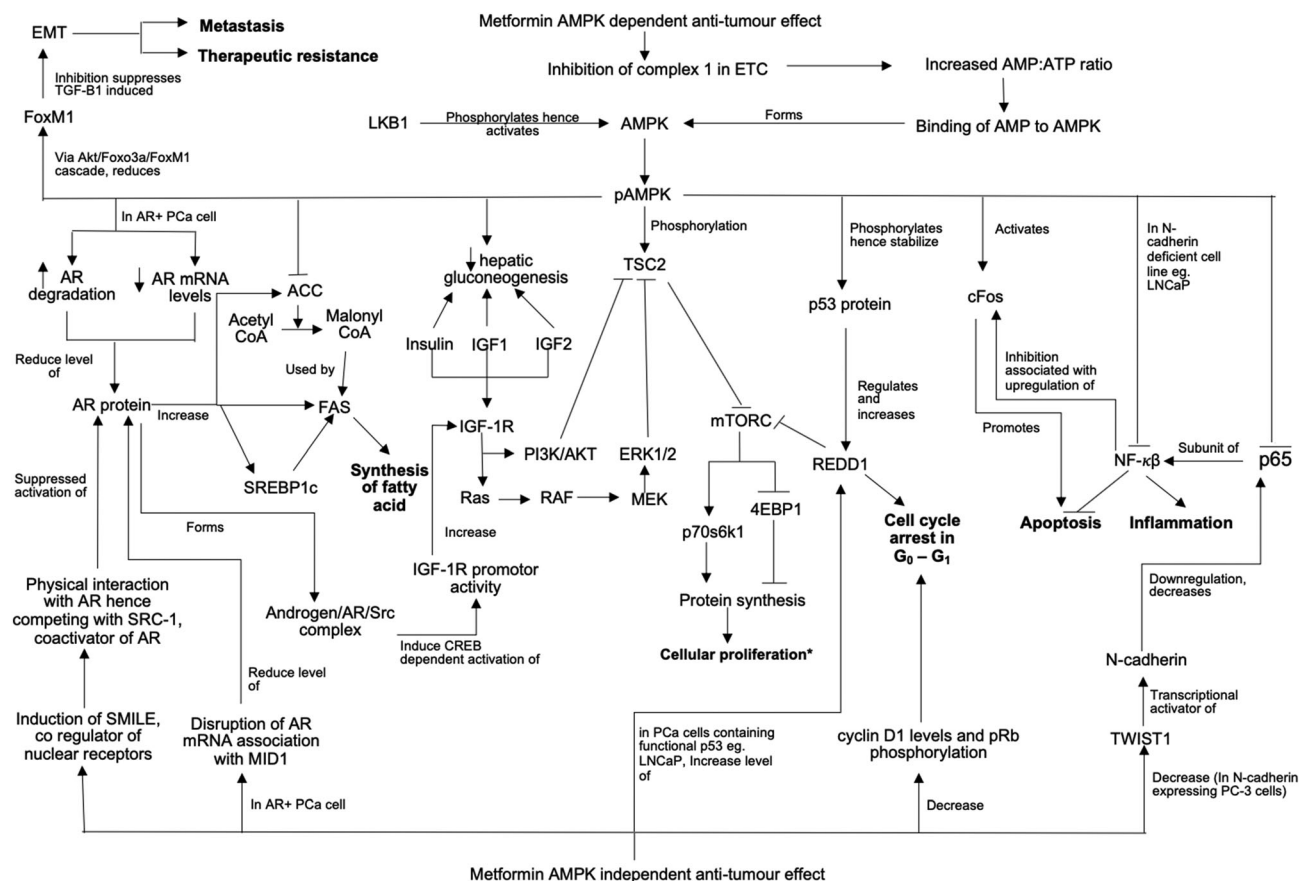
AR signalling drives PCa growth via regulating cellular proliferation and apoptosis[59]; therefore, reducing AR signalling through ADT is the mainstay treatment for locally advanced PCa.[3] Metformin reduces AR signalling in both androgen-independent (LNCaP-abl) and androgen-dependent (LNCaP, C4-2B) PCa cell lines.[39,59] Shen et al. reported AMPK activation by metformin in LNCaP cells resulted in low AR protein levels via reducing AR mRNA expression and increasing AR protein degradation.[59] In contrast, Demir et al. found AMPK activation is not required for the reduction of AR levels.[39] Instead, metformin disrupts AR mRNA association with MID1 translational complex thereby reducing AR protein levels.[39] In addition, Lee et al. reported that metformin increased small heterodimer partner-interacting leucine zipper (SMILE), which serves as an AR corepressor, thereby providing a new mechanism to account for the inhibition of AR function in PCa cells, independent of AMPK activation (Figure 2).[51]

Another key molecular pathway implicated in PCa growth is the type 1 insulin-like growth factor receptor (IGF-1R) signalling cascade. Inhibition of IGF-1R using a human monoclonal antibody successfully inhibited both androgen-independent and -dependent prostate tumour growth in vivo.[81] Activated IGF-1R results in downstream activation of the phosphatidylinositol 3 kinase/protein kinase-B (PI3K/Akt) pathway and mitogen-activated protein kinase (MAPK) pathway thereby increasing cellular proliferation.[82] One paper reported crosstalk between IGF-1R and the AR signalling cascade, with androgen stimulation inducing cAMP-response element-binding protein (CREB) activation and CREB-dependent IGF-1R gene transcription, to upregulate IGF-1R.[58] Metformin is capable of not only downregulating AR as mentioned above, but it can also inhibit androgen-induced cAMP response element (CRE) activity and IGF-1R gene transcription, thereby reducing IGF-1-mediated cell proliferation (Figure 2).[58] The reduction in IGF-1R expression is supported by Kato et al. who showed that IGF-1R mRNA and protein expression decreased following metformin (5 and 10 mM) exposure in PC-3 cells, and that IGF-1R knockdown using siRNA inhibited cellular proliferation, invasion, and migration of PC-3 cells. Daily intraperitoneal injection of metformin in a subcutaneous PC-3 xenograft model reduced tumour growth and IGF-1R mRNA expression which further supports the finding that metformin targets IGF-1R signalling to inhibit PCa growth.[46]

Other AMPK-independent pathways reported to mediate the antitumour effect of metformin in PCa included upregulation of REDD1[50] and reduction of cyclin D1 and pRb phosphorylation inducing cell cycle arrest[40]; in N-cadherin expressing PC-3 cells, metformin reduced TWIST1 thereby silencing N-cadherin and decreasing p65 (subunit of NF-kB) resulting in apoptosis.[44] However, in N-cadherin-deficient LNCaP cells, metformin induced apoptosis via AMPK activation that resulted in inhibition of downstream NF-kB signalling.[44,83] Therefore, the metformin antitumour effect may be mediated via different molecular mechanisms depending on the specific PCa cell line (Figure 2).

Other studies have investigated the antitumour mechanism of metformin without evaluating AMPK (Figure 3).[43,47,61,62,66-69] In one study, metformin inhibited cellular migration and invasion via downregulation of histone methyltransferase multiple myeloma SET

EMT ——→ **Metastasis** / **Therapeutic resistance**

Inhibition suppresses TGF-B1 induced

Metformin AMPK dependent anti-tumour effect

Inhibition of complex 1 in ETC ——→ Increased AMP:ATP ratio

FoxM1

Via Akt/Foxo3a/FoxM1 cascade, reduces

LKB1 —Phosphorylates hence activates→ AMPK ←—Forms—— Binding of AMP to AMPK

In AR+ PCa cell

pAMPK

Phosphorylation

↓ AR degradation   ↓ AR mRNA levels   ACC   ↓ hepatic gluconeogenesis   TSC2   Phosphorylates hence stabilize   Activates   In N-cadherin deficient cell line eg. LNCaP

Acetyl CoA ⇌ Malonyl CoA

Reduce level of

Insulin   IGF1   IGF2   p53 protein   cFos

Used by

AR protein —Increase→ FAS

Suppressed activation of

SREBP1c

Forms

↑ IGF-1R   PI3K/AKT   ERK1/2   mTORC   REDD1   Inhibition associated with upregulation of   Promotes   NF-κB ← Subunit of → p65

Synthesis of fatty acid

Ras → RAF → MEK   p70s6k1   4EBP1

Regulates and increases

Reduce level of

Physical interaction with AR hence competing with SRC-1, coactivator of AR

Androgen/AR/Src complex

Increase

IGF-1R promotor activity

Induce CREB dependent activation of

Protein synthesis

Cell cycle arrest in G₀ – G₁   **Apoptosis**   **Inflammation**

Cellular proliferation*

Induction of SMILE, co regulator of nuclear receptors

Disruption of AR mRNA association with MID1

Reduce level of

In AR+ PCa cell

in PCa cells containing functional p53 eg. LNCaP, Increase level of   cyclin D1 levels and pRb phosphorylation   Decrease

Decrease

Downregulation, decreases

N-cadherin

Transcriptional activator of

TWIST1

Decrease (In N-cadherin expressing PC-3 cells)

Metformin AMPK independent anti-tumour effect

FIGURE 2 Schematic diagram of AMPK-dependent and -independent anti-tumour effects of metformin in PCa. The diagram is a summary of findings from the above 37 articles in "metformin molecular anti-tumour effect". Abbreviations: AMPK, AMP activated kinase; AR, androgen receptor; ERK, extracellular signal regulated kinase; EMT, epithelial to mesenchymal transition; FAS, fatty acid synthase; LKB1, liver kinase B1; mTORC, mammalian target of rapamycin complex; phosphatidylinositol 3 kinase, PI3K; SREBP1c, sterol regulatory element binding protein 1c; TSC2, tuberous sclerosis complex 2.

(MMSET),[66] and inhibition of Rac1.[43] Metformin has also been reported to activate tumour suppressor Foxo3a resulting in cell cycle arrest[67] and may affect tumour-promoting inflammation, as it inhibited infiltration of tumour-associated macrophages via a reduction in COX2 and PGE$_2$ in PCa cells.[47] Finally, many cancer cells source carbon by increasing glucose uptake and lactate from cancer-associated fibroblasts (CAFs).[62] Metformin can reprogram PCa cell metabolism by reducing Glut1 and MCT1 expression in PCa cells, reducing capacity to take up glucose and lactate through these transporters.[62]

3.3 | Quantitative analysis

3.3.1 | In vivo effects of metformin

Metformin was tested in PCa preclinical models. While 13 articles included mouse models, one had an incorrect design and did not include PCa despite their study aims.[72] In the 12 remaining papers that used metformin in PCa mouse models, three papers used models of spontaneous PCa including the TRAMP model[47,55] or a Hi-Myc mouse model (where c-myc was overexpressed under the control of the

probasin promoter)[54] to assess the effect of metformin on prostatic intraepithelial neoplasia and cancer lesion development. Of the remaining nine studies, four PCa xenograft models were used. Three studies used the AR-positive cell line LNCaP[40,44,45] and one study used AR-positive, androgen insensitive 22RV1 cells.[68] Most studies used AR-negative cell lines, PC-3 cells (six studies) or DU145 (one study).[41–46,62] One study injected PC-3 cells orthotopically,[43] and one used injection of PC-3 cells in the lateral tail vein[62] in order to assess metformin effect on metastases. The remaining seven studies utilized subcutaneous xenograft models[40–42,44–46,68] and measured differences in tumour volume after metformin treatment as their primary outcome.

In the seven subcutaneous models, mice were injected with between 1 and 7 million cells per mouse, although the majority used 1–2.5 million cells. Metformin dose was given daily in drinking water (doses ranging from 100 to 250 mg/kg/day),[40,41,43–45,47,54] gavage (300 mg/kg),[68] intraperitoneal injection (doses ranging from 20 to 250 mg/kg/day),[40,42–44,46,55] and in one case, PC-3 cells were pre-treated with conditioned media from cancer-associated fibroblasts grown in media with or without 5 mM metformin, before injection and monitoring of tumour growth without in vivo metformin treatment.[62]
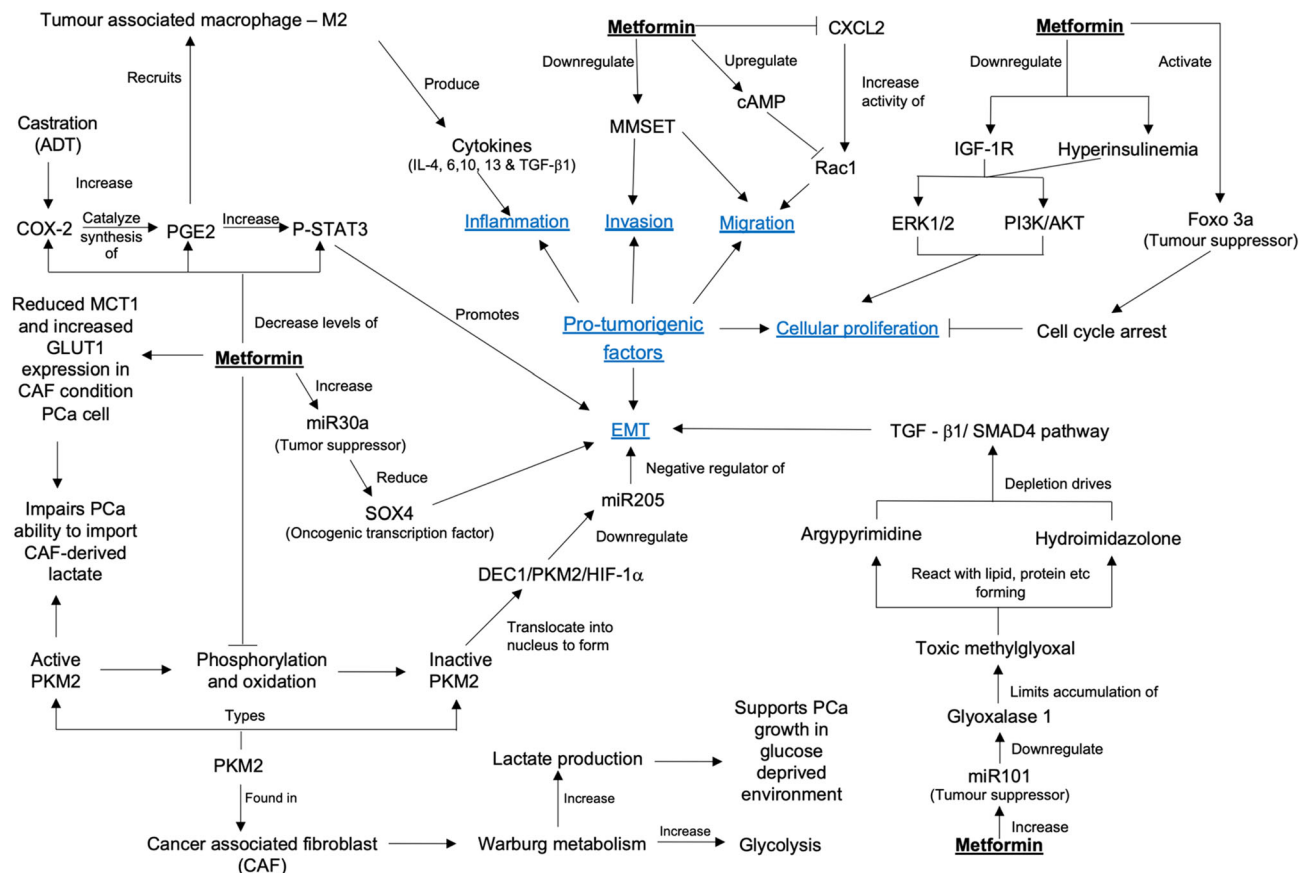
FIGURE 3 Schematic diagram of other molecular mechanisms accounting for the anti-tumour effect of metformin in PCa. The role of AMPK in these mechanisms have yet been investigated.

Universally, metformin was reported to reduce tumour initiation, with reduced prostatic intraepithelial neoplasia lesions,[47,54] reduced metastases consistent with decreased expression of c-myc and EMT hallmark genes,[54,62] and reduced tumour growth for both subcutaneous and intraprostatic xenograft PCa models.[40–47,54] To further evaluate the reported effects of metformin on subcutaneous tumour growth, we performed a meta-analysis of studies where tumour volume was reported. This limited our meta-analysis to include eight of the 11 in vivo studies with treatment duration 4–6 weeks (Table S5)[40–47] and demonstrated a statistically significant drug effect reducing tumour growth (Figure 4A, weighted SMD −1.81, overall effect $Z = 5.74$, $p < 0.00001$), with low study heterogeneity ($I^2 = 39\%$). Given this strong association, publication bias was assessed by funnel plot (Figure 4B), which demonstrates a shift from the expected mean indicating publication bias for studies showing metformin as an inhibitor of prostate tumour growth in these model systems.

4 | DISCUSSION

T2DM is associated with an increased risk of a variety of cancers.[20,21,84] Metformin is the first-line pharmacotherapy for T2DM, used by at least 120 million people worldwide[14] and has low toxicity.[15] Various epidemiological studies have shown that diabetes patients treated with metformin had reduced risk of PCa,[17–19] with improved survival outcomes.[23,24] However, direct clinical evidence of tissue-specific activity is lacking, and it has been suggested the effects of metformin on cancer outcomes could be due to the systemic effect of metformin rather than direct antitumour effects. Two meta-analyses have indicated that metformin is associated with reduced biochemical recurrence as compared with nonusers in T2DM patients with PCa.[28,35–38] The underlying mechanism in PCa are multifactorial. Herein, we review and summarize the efficacy and mechanism of action of metformin as an antitumourigenic agent in PCa.

The 15 in vitro studies demonstrate metformin is capable of reducing androgen-dependent PCa cell growth,[39–42,46,50–52,55,58–61,64,67] while 17 studies concluded that metformin inhibited the viability of androgen-independent PCa cell growth,[39,40,42,44,46,47,50–55,59–61,66,67] suggesting that metformin could be combined as anticancer therapy at various stages of PCa. The inhibitory effect of metformin on PCa cellular growth is replicated in vivo where metformin suppresses PCa (androgen-independent and androgen-dependent) tumour growth in multiple studies.[40–47]

The molecular mechanisms by which metformin inhibits PCa growth are highly complex (Figures 2 and 3) and multimodal. AMPK is a master regulator of homeostasis that has a controversial role. While AMPK was identified as a tumour suppressor initially,[85–88] multiple

(A)

Author	Mean	Std Dev	n/group	Mean	Std Dev	n/group	Weight
Sarmento-cabral (2017b)	75	5	6	240	15	5	0.7%
Liu (2018)	1000	250	3	4000	400	3	0.7%
Hirsch et al (2012b)	125	44	3	625	68	3	0.8%
Ge et al (2015c)	267	248	5	1266	199	5	4.4%
Hirsch et al (2012a)	433	33	3	600	35	3	2.0%
Kato et al (2015)	500	125	5	725	30	5	7.5%
Chen et al (2016b)	360	80	5	1000	400	5	8.0%
Ge et al (2015a)	500	224	5	1150	402	5	8.4%
Ge et al (2015b)	800	244	5	1200	161	5	8.5%
Ben Sahra (2007a)	280	18	5	620	335	5	8.9%
Dirat et al (2015b)	140	99	8	315	147	6	11.0%
Ben Sahra (2007b)	142	7	5	203	49	5	9.4%
Ge et al (2015d)	910	199	5	1190	248	5	9.7%
Chen et al (2016a)	610	200	5	1000	400	5	9.7%
Sarmento-cabral (2017a)	165	30	6	200	35	5	10.4%
Total			**74**			**70**	**100%**

Heterogeneity: Tau^2=0.52; Chi^2=22.98; df=14 (p=0.06); I^2=39%
Test for overall effect: Z 5.74 (p<0.00001)

(B)
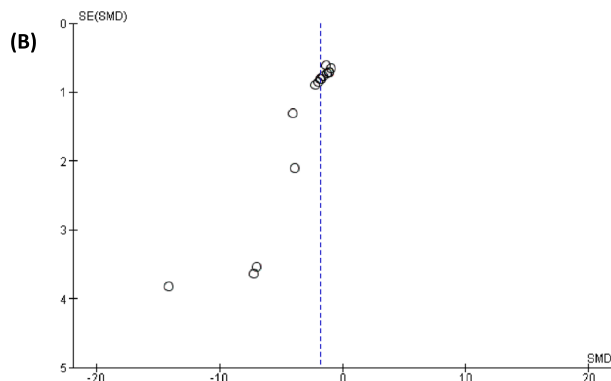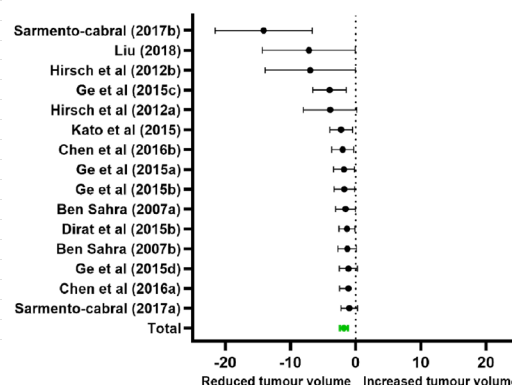
FIGURE 4 Meta-analysis of the effect of metformin on growth of subcutaneous prostate cancer tumours. (A) The combined data shows a significant effect of metformin in tumour reduction. Group size and nature of the data comparison results in high degree of heterogeneity. Dirat[43] was excluded as the study duration was 2 weeks and the remaining studies were 4–6 weeks duration. (B) Funnel plot analysis shows a deviation from the expected mean indicating publication bias with published in vivo studies showing an overwhelmingly inhibitory effect of metformin on tumour growth.

studies have also reported a protumourigenic role.[89–91] Several AMPK-dependent[44,59,60] and AMPK-independent pathways[39,40,44,51] for metformin activity have been identified in this study; however, not all studies evaluated whether AMPK was involved in the antitumour effect of metformin.[43,47,61,62,66–69] Regardless of the mechanisms, most of the studies included in this review showed an antitumour role of metformin. This is mediated in a variety of ways including inhibition of cell cycle progression,[40,50,54,58,61,66] induction of apoptosis,[42,44,47,52,54,59] inhibition of EMT,[60–62,68,69] blocking cellular invasion and/or migration,[39,41–43,46,66,68,69] reducing tumour-enabling inflammation,[47] or changing metabolism.[62,63] Mechanistically, the two most activated oncogenic signalling cascades are the AR and PI3K/AKT signalling pathways. These two pathways cross regulate each other such that the inhibition of one pathway activates the other to allow PCa survival. Hence, combined pharmacological inhibition of these two pathways resulted in complete regression in PCa xenografts.[92] Metformin downregulates IGF-1R expression hence reducing downstream activation of AKT,[46,58] whereas ARSIs or ADT reduces AR signalling,[3] therefore the combination of both could potentially inhibit the cross regulation of the two main oncogenic signalling pathways driving PCa growth.

ADT, with sequential addition of ARSI are the mainstay treatment for advanced PCa.[3,12] However, it has been shown that castration or ARSIs could induce EMT,[13,68] a process underlying metastasis and therapeutic resistance.[68] Metformin inhibited castration-induced EMT and reversed ARSI (e.g., enzalutamide) resistance, thereby suggesting that the combination of metformin with ADT or ARSI could be a possible therapeutic option for advanced PCa.[13,68] Moreover, in vitro PCa studies that evaluated the combination treatment of an ARSI (bicalutamide) with metformin showed greater growth inhibition in the combination treatment as compared with either agent used alone.[52,59,93] Liu et al. evaluated the effect of an ARSI (enzalutamide) with metformin in PCa xenografts in vivo, showing that the combination treatment also had a greater inhibition on tumour growth than metformin alone, although in that study enzalutamide on its own had no effect.[13] These data support the idea that metformin in combination with ADT or ARSI may be a potential therapeutic option for PCa patients. In a recent randomized controlled trial (RCTs) of patients with advanced PCa, the addition of metformin to standard of care resulted in improved cancer-free survival for both patients with high risk localized disease and patients with metastatic low tumour volume disease.[94]

Metformin also appears to have a role in secondary prevention of PCa. Five meta-analyses conclusively indicated that metformin reduced biochemical recurrence (BCR) of PCa,[28,35–38] highlighting the possibility of repurposing this antidiabetic drug as an antitumour agent in PCa of various stages (Table 2). Until recently, no RCT have been done to ascertain the effect of metformin in low risk PCa.[95] However, given the promising data seen in preclinical and clinical studies in the last decade, the Metformin Active Surveillance Trial (MAST) has been set up to evaluate the role of metformin in reducing progression amongst men on expectant management for low risk PCa. This is a phase III randomized, double-blinded, placebo-controlled trial that is ongoing and estimated to be completed by 2024.[96]

4.1 | Strength and limitations

The strength of this study lies in the comprehensive search strategy, which included as many articles as possible, therefore providing a holistic overview of the mechanisms and antitumour effect of metformin in PCa. Moreover, all included articles ($n = 35$) have been assessed for data reliability, with the majority being classified as category 1 articles; hence, the findings are reliable.

Nonetheless, this study has several limitations. First, for the in vivo findings, all but one article showed inhibition on PCa tumour growth, suggesting the following possibilities—(1) metformin indeed is potent in terms of inhibiting PCa tumour growth in mice models; or (2) nonsignificant results are rarely published in this field, such that most of the published findings are skewed towards the positive side. Secondly, most of the studies published identified a novel mechanism, and these findings have not yet been verified by other in vitro or in vivo studies. Thirdly, the concentration for metformin used for the in vitro studies were much higher than plasma concentration of metformin in diabetic patients.[76] Most of the experiments used a concentration of 1–50 mM (Table S5), whereas the plasma concentration of patients taking metformin varies between 0.3 and 1.3 mg/L and maximum obtainable concentration of 3 mg/L, which equates to 1–20 μM.[76,97] Hence, whether there is an antitumour effect seen at therapeutically relevant plasma concentrations of metformin has not been determined. However, it is important to note that metformin accumulates across the mitochondrial matrix to target the prostate tumour energetics directly. The concentration of metformin in prostate tissue has been shown to be approximately two to 32 times higher than that in serum of PCa patients.[74]

In addition, in vitro studies are generally not conducted to take into consideration the pharmacokinetics of metformin, where peak plasma concentrations occur 2 h post-dose and the elimination half-life in patients with good renal function is approximately 5 h.[76] In addition, all in vitro experiments reviewed were conducted over experimental time frames of 24–96 h and at concentrations an order of magnitude higher than found in plasma. This discrepancy is a source of controversy in the field. With limited evidence of tissue-specific concentrations,[76] particularly over sustained treatment periods,[98] even with adequate controls, differences in effective metformin dose are difficult to compare and may be a function of cancer cell type and predominant metabolic (glycolytic or oxidative) and glucose addiction.[99] Future in vitro studies could better recapitulate therapeutic plasma concentrations of metformin at steady state observed in T2DM patients. This may remove half-life as a confounding factor until further studies of intratumoural concentrations of metformin can be ascertained over sustained treatment times.[98]

4.2 | Conclusion

In conclusion, this systematic review summarizes the in vitro antitumour effects of metformin in PCa and meta-analysis on the inhibition of PCa growth in mouse models by administration of metformin. The findings from preclinical studies support the idea that metformin could be repurposed as an anticancer therapeutic option in PCa either alone or in conjunction with current therapies (ADT or ARSIs). This is based on the following factors: (1) The ability to inhibit PCa growth in vitro and in vivo, (2) reduce ADT-induced cardiovascular adverse effects, and (3) reversing castration- or treatment-induced EMT.

4.3 | Future directions

Given the meta-analysis revealed publication bias in the in vivo studies, more preclinical data may be needed to ascertain the antitumour effect of metformin in PCa mouse models. In addition, the majority of the clinical studies that evaluated the clinical outcome of PCa patients who are on metformin are retrospective in nature, which has inherent biases,[29] such as selection bias, interviewer bias or recall bias. Thus, more long-term RCTs are needed to ascertain whether the inhibitory effect of metformin on PCa growth in preclinical studies are applicable to PCa patients. This is a rapidly evolving field, with the first RCT being published last year showing that metformin combination with ADT improves the outcome for patients with advanced PCa.[94] While the MAST (a randomized, double-blinded, placebo-controlled trial), which looks at the role of metformin in early stage PCa, is currently ongoing and will be completed in 2024.[96]

CONFLICTS OF INTEREST

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work, no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years, and no other relationships or activities that could appear to have influenced the submitted work.

AUTHOR CONTRIBUTION

NFW and JH conceptualized the review, NFW performed the literature search, and NFW and JHG undertook independent review, quality scoring, and meta-analysis. NFW, TRJ, JH, and JHG wrote the article, and JH and JHG are guarantors of the manuscript. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

TRANSPARENCY STATEMENT

The guarantors affirm that our manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as originally planned (and, if relevant, registered) have been explained. This review was not registered. All protocols are presented with this submission.

PATIENT AND PUBLIC INVOLVEMENT

Patient and public involvement was not engaged for this study.

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